

## Classic methods revisited

# Widal agglutination test – 100 years later: still plagued by controversy

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### Summary

**We review the significance of the Widal agglutination test in the diagnosis of typhoid fever. Over 100 years since its introduction as a serologic means of detecting the presence of typhoid fever, the Widal test continues to be plagued with controversies involving the quality of the antigens used and interpretation of the result, particularly in endemic areas. Areas of concern with clinical and laboratory significance discussed in this review include: the techniques of test performance, interpretation of results, limitation of the value of the test results in endemic typhoid areas, the quality of the antigens used, and alternative diagnostic tests.**

**Keywords:** Widal agglutination test; typhoid fever

Agglutination is a classic serologic reaction that results in clumping of a cell suspension by a specific antibody, directed against a specific antigen. Such tests have been widely used for detection of antibodies against various disease-producing micro-organisms in serum for a long time. The Widal agglutination test, developed by F Widal in 1896<sup>1</sup> to aid in the diagnosis of typhoid fever, utilises a suspension of killed *Salmonella typhi* as antigen, to detect typhoid fever in serum from suspected *S typhi*-infected patients who present with febrile illness. The value and clinical application of the Widal test in developed countries has diminished considerably in recent years<sup>2</sup> and a large number of antigenically related determinants of both typhoid and non-typhoid *Salmonella* organisms are now recognised. We therefore decided to review the significance of this sero-diagnostic test for typhoid fever in modern medicine, and to discuss new and innovative alternative diagnostic tests. Hopefully, this review will offer both the novice and the experienced physician the opportunity to appreciate the limitations of the Widal test.

### Widal agglutination

Widal agglutination was introduced as a serologic technique to aid in diagnosis of typhoid fever. The test was based on demonstrating the presence of agglutinin (antibody) in the serum of an infected patient, against the H (flagellar) and O (somatic) antigens of *Salmonella typhi*. While the definitive diagnosis of typhoid fever depends on the isolation of *S typhi* from blood, stools, urine or other body fluids,<sup>3–5</sup> the role of the Widal test had been to increase the index of suspicion for the presence of typhoid fever by demonstrating a positive agglutination during the acute and convalescent period of infection with evidence of a four-fold rise of antibody titre.<sup>6–8</sup> In developed countries, the use of Widal agglutination as a laboratory tool to aid in the diagnosis of typhoid fever during the acute phase of the illness, has largely been abandoned,<sup>2</sup> as the need for such a test is minimal, especially in view of the low prevalence of typhoid fever. In addition, adequate and improved sanitation, sewage systems, proper hygiene and better means of isolating the organism from culture are available. Unfortunately, in some developing countries, the situation is quite different, and the Widal test appears to be the only laboratory means employed in the diagnosis of typhoid fever among suspected patients. As the test suffers from serious cross-reactivity with other infectious agents, it may produce false-positive results, leading to an over-diagnosis of typhoid fever. Reynolds *et al*<sup>9</sup> concluded that diagnosis of typhoid fever based on serology (Widal agglutination) alone is frequently inaccurate. Concomitant with this increase in diagnosis is the abuse of the first-line drug of choice (chloramphenicol), which has led to the selection of resistant strains of *S typhi*.

### Performance technique

The Widal test reaction involves the use of bacterial suspensions of *S typhi* and *S paratyphi* 'A' and 'B', treated to retain only the 'O' and 'H' antigens. These antigens are employed to detect corresponding antibodies in the serum of a patient suspected of having typhoid fever. The IgM somatic O antibody appears first and represents the initial serologic response in acute typhoid fever, while the IgG flagella H antibody usually develops more slowly but persists for longer.<sup>3 8 10</sup>

Two types of agglutination techniques are available: the slide test and the tube test. The slide test is rapid and is used as a screening procedure. Using commercially available antigens of *S typhi*, a drop of the suspended antigen is added to an equal amount of previously prepared serum. An initial positive screening test requires the determination of the strength of the antibody. This is done by adding together equal amounts of antigen suspension and serially diluted serum from the suspected patient. Agglutinations are visualised as clumps. Weakly

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reactive agglutinations may require an adequate light source for proper visualisation, while strongly reactive agglutinations are easily seen. The result of the tests are scored from 0 to 4+, ie, 0 (no agglutination), 1+ (25% agglutination), 2+ (50% agglutination), 3+ (75% agglutination) or 4+ (100% agglutination). The smallest quantity of serum that exhibits a 2+ or 50% agglutination is considered the end-point of serum activity or titre.

The tube agglutination test requires much more technical work than the rapid slide test, and is a macroscopic test.<sup>11 12</sup> It also serves as a means of confirming the results of the slide test. A mixture of suspended antigen and antibody is incubated for up to 20 h at 37°C in a water bath. Agglutinations are visualised in the form of pellets, clumped together at the bottom of the test tube. Results are scored from 0 to 4+ positive agglutination as described above for the slide test. The tube test is useful to clarify erratic or equivocal agglutination reactions obtained by the more rapid slide test.

Since the ultimate goal of the test is antigen–antibody complex reaction, cross-reactions are encountered when antibody produced by non-typhoidal antigens reacts with typhoid-specific antigens. Several other diseases caused by non-*Salmonella* organisms (malaria, dengue, miliary tuberculosis, endocarditis, chronic liver disease, brucellosis, etc) have been shown to exhibit this cross-reactivity in typhoid endemic regions, and these cross-reactions increase the error rate of the result of the Widal test.

Lack of standardisation of antigens also compromises the technique, as shown by Devillier *et al.*<sup>13</sup> The value of Widal test depends upon the standardisation and maintenance of the antigens to produce consistent results, and it has become evident from work done in recent years on standardisation of the Widal test and interpretation of the results that both the O and H antigens are necessary for proper serologic analysis of the suspected serum. However, according to Welch in 1936,<sup>11</sup> no Widal test, regardless of the composition and standardisation of the antigens used, is infallible, and thus it is unlikely that any will be developed that will lower the validity of the isolation of the aetiologic agent. Unfortunately, more than 60 years after Welch published his paper, the problems of ambiguity, insensitivity and non-specificity of Widal antigens continue. The widespread use of typhoid–paratyphoid vaccine, as well as the large number of cases of repeated exposure to *Salmonella* species, tend to lower the specificity of the Widal test. We consider that serologic studies are helpful in typhoid fever cases in endemic regions only if patients have four-fold or greater increases in O or H agglutinin titres in serum specimens obtained 2–3 weeks apart.

### Interpretation of the test results

While performance of the test may require some detailed technical work, interpreting the test result is the more arduous task. *Salmonella* are divided into distinct serologic groups (A through E) on the basis of their somatic O antigens. While all group D organisms, such as *S typhi* possess O antigen 9, about 60 of the 78 group D serotypes including *S typhi* also have O antigen 12.<sup>14</sup> Thus, infection by any of the group D serotypes can produce antibodies that can react with the O antigen used in the Widal reaction. Also, since all groups A and B organisms possess O antigen 12, cross-reactions with O antibody of group D serotype can occur with any of the group A and B serotype O antigens. Depending on the relative quality and quantity of antigenicity of the O antigens 9 and 12 contained in other common non-typhoidal *Salmonella* serotypes, cross-reaction may occur frequently enough to lessen considerably the diagnostic specificity of the Widal reaction. A comparative study of *S typhi* O antigen obtained from different manufacturers tested against the same serum, which had previously been shown to be positive by the slide agglutination test, revealed marked variability associated with the Widal agglutination titre.<sup>13</sup> A negative agglutination test may be for one of several reasons, given in box 1. A negative Widal test result does not therefore necessarily rule out the absence of infection. Such results are best kept as a reference for subsequent comparative analysis.

A positive agglutination tests (on two successive occasions) on the other hand, may also be open to several different interpretations (box 2).

Although there are controversies surrounding the increase in titre beyond the first week of illness in some endemic areas, it is generally accepted by clinicians that, toward the end of the first week of illness, titres of either O or H antibody may rise to as high as 1:160. However, the lack of paired sera may lead to an erroneous interpretation of test results.<sup>10 15</sup>

In endemic typhoid regions, a single testing of a serum specimen for Widal agglutinin cannot provide a reliable diagnosis due to:

- repeated exposure to small inocula of *S typhi* or to other *Salmonella* spp that contain type 9 or 12 antigens

#### Causes of negative Widal agglutination tests

- absence of infection by *S typhi*
- the carrier state
- an inadequate inoculum of bacterial antigen in the host to induce antibody production
- technical difficulty or errors in the performance of the test
- previous antibiotic treatment
- variability in the preparation of commercial antigens

Box 1

#### Causes of positive Widal agglutination tests

- the patient being tested has typhoid fever
- previous immunisation with *Salmonella* antigen.
- cross-reaction with non-typhoidal *Salmonella*.
- variability and poorly standardised commercial antigen preparation
- infection with malaria or other enterobacteriaceae
- other diseases such as dengue

Box 2

**Table** Widal agglutinin titres in Nigerian patients with or without a positive malaria smear

Group	Patients (n)	1:40	1:80	1:160
1 *(PMS, NSTC, LPTI)	45	85%	12%	3%
2 *(NMS, NSTC, LPTI)	69	45%	15%	10%

\*PMS = positive malaria smear; NMS = negative malaria smear; NSTC = negative *S typhi* culture; LPTI = lack of prior typhoid immunisation.

- previous typhoid fever immunisation
- other infectious agents such as malaria.

Although a number of reports from some developing countries have suggested that a single Widal test is sufficient to make the diagnosis of typhoid fever,<sup>16-20</sup> others have disputed the usefulness of such a single test result.<sup>9 10 14 15 21 22</sup> In some developing countries where the use of a single Widal test appears to be the norm, there has been an increase in the rate of false-positive results. We have studied Widal agglutinin in malaria infection in a Nigerian population and found that 85% of patients with a negative *S typhi* culture but positive malaria smear had Widal titres of 1:40, 12% had titres of 1:80, and 3% had titres of 1:160. In contrast, 45% of patients with both *S typhi* cultures and malaria smears negative had Widal titres of 1:40, 15% had titres of 1:80, and 10% had titres of 1:160 (table). Schroeder<sup>23</sup> concluded in a review of clinical interpretation of serologic tests for typhoid fever that the tests are nonspecific, poorly standardised, confusing and difficult to interpret. Erroneous interpretation of the test result may lead to misdiagnosis and mismanagement of the patient, resulting in major morbidity and mortality.

In interpreting Widal test results, it is important that there should be close communication between the physician requesting the test and the laboratory, since modifications of technique in individual laboratories may affect the Widal titres and some patients with bacteriologically confirmed typhoid fever may fail to develop the usual rise of antibody titres. The results of the tests should be reported as either 'no agglutination' or, if agglutination is present, in titres (1:20, 1:40 or 1:80) rather than in descriptive (negative or positive) terms, as the latter may be misleading and contribute to the false interpretation of the test result by the physician. The function of the laboratory is to perform and report the test result to the requesting physician, who in turn will use the data to help make the proper diagnosis. Unfortunately, in several areas of developing countries, the laboratory performs the test, makes the diagnosis and prescribes the antibiotics.

It should be stressed that a single Widal agglutination test has no diagnostic significance. According to Hoffman *et al.*,<sup>10</sup> the results of a single Widal test, tube dilution, micro-agglutination or slide agglutination are virtually un-interpretable unless the sensitivity and specificity of the test for the specific laboratory and patient population are known, as well as predictive values. Even in the extreme case of a high titre in a single Widal agglutination test, the causative organism may often be due to other species of *Salmonella*, rather than *S typhi*. Sansone *et al.*<sup>6</sup> published a case report where the Widal reaction to typhoid O antigen on admission for an unexposed patient was 1:320, with an increase in titre to 1:20 480 by the fourth day. While both blood and urine cultures were negative for *S typhi* in this case, a non-typhoidal *Salmonella* sp was isolated from the stool of this patient which was identified as *S javiana*. In an individual with no prior exposure to *S typhi* infection (either lack of active infection or absence of passive immunisation), a higher than 1:50 or 1:100 titre on an initial single test, usually correlates fairly well with exposure to typhoid fever. However, even these single high-value titres in an endemic area where repeated exposures to *S typhi* may have occurred, do not have any clinical relevance in the absence of a positive isolate of the causative organism or its antigen.<sup>11</sup>

### Limitations of the Widal test

While the Widal test has played a major role in the diagnosis of typhoid fever in the past, recent technical developments have revealed several pitfalls in its use and interpretation of its result. Clinically, it is obvious that a single Widal test in an unvaccinated or unexposed patient may have some diagnostic relevance. However, the result of such a single test has no diagnostic significance in an endemic region; in part due to difficulty in establishing a steady-state or baseline titre of Widal agglutination, which limits the usefulness of the test as a reliable diagnostic indicator of the disease process.

The results of studies done in Nigeria to evaluate the clinical value of a single Widal test and the presence of Widal agglutinin in malaria infection are

summarised in the table.<sup>15 24</sup> The common denominator between the two groups was the lack of prior immunisation against typhoid fever and absence of positive *S typhi* culture. One would not therefore expect any patient to have any specific Widal agglutinin in their serum, unless there are related, undetected, antigenic determinants of *S typhi* present in the cells of other organisms. The presence of Widal agglutinin under conditions of positive malaria smear, negative *S typhi* culture and negative prior typhoid immunisation (as seen in group 1), would suggest that malaria parasite may have some undefined antigenic determinants similar to *S typhi* which can induce antibody production. This could explain the febrile condition seen in some of these patients. On the other hand, the presence of Widal agglutinin under conditions of negative malaria smear, negative *S typhi* culture and negative prior immunisation against typhoid fever (as seen in group 2) suggests that other infectious agents, in addition to *Salmonella* and malaria parasite, may also share common antigenic determinants with *S typhi*. These findings are in agreement with other reports from India with similar environmental and disease (malaria, typhoid) conditions,<sup>25-31</sup> two cases from Canada,<sup>32</sup> and a case from Baltimore,<sup>33</sup> all of which cast further doubt on the reliability and the use of Widal test for the diagnosis of typhoid fever in endemic regions.

The use of the Widal test to diagnose typhoid fever should therefore be limited to situations in which there is no other confirmatory supportive test, such as positive culture, available. Similarities between typhoidal and non-typhoidal *Salmonella* antigens mean that a serological method of diagnosis is the least accurate for typhoid fever. Due to the inexperience of some clinicians in typhoid endemic countries, many cases of pyrexia of unknown origin receive the diagnosis of typhoid fever, based upon a false-positive Widal test result rather than a positive culture of *S typhi*.

### Antigen detection as an alternative to Widal agglutination

While bacteriological culture remains the gold standard for definitive diagnosis of typhoid fever, lack of its immediate availability during the acute febrile illness may limit its use. In an acute febrile illness in an endemic typhoid region where the clinical picture is ambiguous, a rapid, accurate, specific and sensitive test should be used to differentiate typhoidal from non-typhoidal febrile illnesses. Clinicians usually elect to treat, rather than wait for blood or stool culture results, which may take 3–5 days. While there might be some merit in this approach, particularly in areas where culture facilities are either poor or not available, and where Widal testing is the norm, the use of rapid antigen screening directly from the stool of the suspected patient would be more useful.

Khan *et al*<sup>34 35</sup> have described a new rapid immuno-enzymatic dipstick test for detection of *Salmonella* directly from the stool. The test which is non-invasive, involves homogenisation of stool sample in a buffer solution and immersion of a dipstick (previously coated with antibodies) in a tube containing the supernatant from the homogenised stool samples. The contents of the tube (dipstick and supernatant) are incubated at room temperature for 15 min and a second tube is incubated for an additional 5 min for full development of colour. The dipstick is air dried and the result is visualised as a horizontal mark on the dipstick. While this test is new, Khan *et al* have reported a preliminary sensitivity of 94%, specificity of 98%, negative predictive value of 99.5%, and positive predictive value of 74%. A large-scale field trial is underway to determine the true sensitivity, specificity and the predictive values. It is hoped that such a direct stool testing will be a useful discriminating test which can be used with confidence in areas where both malaria and typhoid may have similar clinical presentations.

### Conclusion

More than 100 years after the introduction of the Widal test for diagnosis of typhoid fever, the controversy that surrounded the test has not abated. It has become increasingly obvious that bacterial agglutination systems (particularly Widal), while offering a simple methodology, often result in misleading information because of the polyvalent nature of the antigens involved. Whereas cross-reacting antigens are widely distributed in the microbial world, the specificity and sensitivity of bacterial agglutination is not sufficient when used in human serum assays. We believe that Widal test cannot be expected to give a reliable diagnostic result in endemic regions for the following reasons:

- the inherent variabilities of the test
- difficulty in establishing a steady-state baseline titre for the population
- repeated exposures to *S typhi* in endemic regions
- cross-reactivities with other non-*Salmonella* organisms
- lack of reproducibility of the test result.



The use of Widal agglutination should not be encouraged, given all these negative points. As cultures are time consuming, increased efforts should be made to find a better, more rapid, sensitive and specific test (such as antigen screening) to supplement clinical and culture data.

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